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(71) Applicant: **MERCK & CO. INC.**  
**126, East Lincoln Avenue P.O. Box 2000**  
**Rahway New Jersey 07065(US)**

(72) Inventor: **Thomas, Kenneth A.**  
**245 Washington Avenue**  
**Chatham New Jersey 07928(US)**

(74) Representative: **Abitz, Walter, Dr.-Ing. et al,**  
**Abitz, Morf, Gritschneider, Freiherr von Wittgenstein**  
**Postfach 86 01 09**  
**D-8000 München 86(DE)**

(54) **Brain-derived growth factor.**

(57) Brain-derived acidic fibroblast growth factor, (aFGF), is an active mitogen for vascular endothelial cells in culture and is useful for growth of such cultures for coverage of polymeric vascular grafts; growth of such cultures on tubular supports for production of blood vessels for implantation.

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TITLE OF THE INVENTIONBRAIN-DERIVED GROWTH FACTORSUMMARY OF THE INVENTION

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Brain-derived acidic fibroblast growth factor, (aFGF), is an active mitogen for vascular endothelial cells in culture and is useful for growth of such cultures for coverage of polymeric vascular grafts; growth of such cultures on tubular supports for production of blood vessels for implantation; and stimulation or facilitation of blood vessel growth and repair in vivo.

BACKGROUND OF THE INVENTION

The brain-derived aFGF purification and wound healing activity of this invention was described in U.S. Patent 4,444,760, issued April 24, 1984 to Kenneth A. Thomas, Jr. The protein is also described by Thomas et al., Proc. Natl. Acad. Sci., USA 81, 357-361 (1984). The same or similar proteins

may also be present in other partially purified extracts from the central nervous system and other organs. See, for example, Maciag et al., Science, 225, 932 (1984).

5           Although the growth of vascular endothelial cells was accomplished in the past using very high concentrations of fetal calf or adult bovine serum (10-30%) the results were variable, depending on the particular lot of calf serum, and the rate of cell  
10 growth was generally slow. Now, with the present growth factor rapid growth rates are achieved with serum levels from 0 to 2%.

          This novel method of reproducible stimulation of vascular endothelial growth, mediated  
15 by pure protein mitogens permits:

- 1) Covering synthetic polymeric vessels with non-thrombogenic vascular endothelial cells from the host animal, including human, whereby many or all of the clotting problems associated with synthetic  
20 vessel grafts are obviated;
- 2) production of vessels in vitro by growth of host vascular endothelial cells on tubular supports, for implantation back into the same host animal, including human, whereby immunological rejection of  
25 the implant will be obviated and the frequent limited supply of good vessels within the patient for transplant will also be obviated; and
- 3) stimulation or facilitation of blood vessel growth and repair in vivo, whereby the flow of blood  
30 to tissues deprived of adequate oxygen and/or other blood borne components is increased.

DETAILED DESCRIPTION OF THE INVENTION

The brain-derived aFGF useful in the novel methods of this invention is prepared as described in U.S. Patent 4,444,760 the disclosure of which is  
5 incorporated herein by reference.

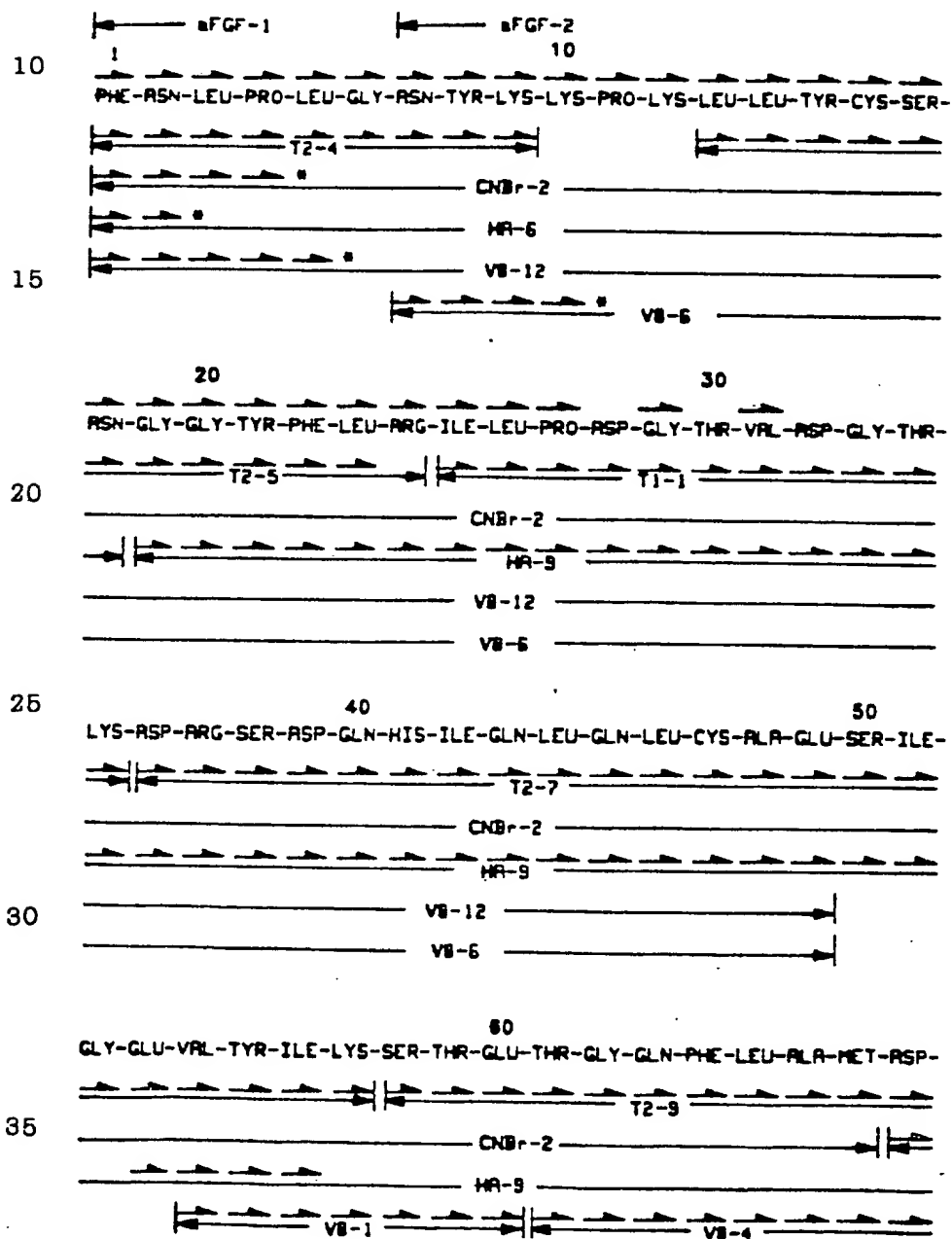
The complete amino acid sequence has been determined for aFGF. Sequence determinations of reduced and carboxymethylated protein have revealed two amino termini. The longer sequence, aFGF-1,  
10 contains six amino terminal residues not found on the shorter aFGF-2 form. The relative amounts of these two microheterogeneous forms of the mitogen vary from one purification to another but are closely correlated in amount to the abundance of the two  
15 bands of protein previously seen by electrophoresis in SDS polyacrylamide gels (Thomas, et al., Proc. Natl. Acad. Sci. USA, 81, 357 (1984)). As expected, the amount of the longer amino terminal sequence correlates with the relative quantity of the higher  
20 mass band on the SDS gels. If the length of the polypeptide chain at the amino termini is the only difference between the two microheterogeneous forms observed on the SDS gels, then the mass difference between them is 642 daltons, rather than the  
25 previously estimated 200 daltons based on SDS gel migration distances. It is assumed that the amino terminal heterogeneity is the result of limited proteolysis either in vivo or during purification.

The complete amino acid sequence was  
30 determined from sequences of the amino termini and overlapping peptides generated by proteolytic cleavages with trypsin (T), Staphylococcus aureus V8 Protease (V8), hydroxylamine (HA) and cyanogen-bromide

- 1 (CN). The carboxyl terminal sequence of the whole protein was confirmed by timed carboxypeptidase A digestion.

- 5 In a search of the current Dayhoff protein data bank, aFGF is unique compared to the approximately 2000 protein sequences contained in that list.

The sequence is as follows:



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1           Peptide sequences that were prematurely  
terminated because they were recognized to begin at  
one of the two previously determined amino termini are  
marked with asterisks following the last degradation  
5 cycle.

The novel method for the stimulation of  
vascular endothelial cells comprises treating a  
sample of the desired vascular endothelial cells in a  
nutrient medium with aFGF at a concentration of about  
10 1-10 ng/ml.

If the vascular endothelial cell growth is  
conducted in vitro, the process requires the presence  
of a nutrient medium such as Dulbecco's modified  
Eagle's medium or modification thereof and a low  
15 concentration of calf or bovine serum such as about 0  
to 2% by volume. Preservatives such as a penicillin-  
streptomycin combination or other broad spectrum  
antibacterials are also employed. It is preferred to  
have about 10 to 100 ug/ml of heparin present also.

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The novel method of this invention is useful for the coverage of artificial blood vessels with endothelial cells. Vascular endothelial cells from the patient would be obtained by removal of a small  
5 segment of peripheral blood vessel or capillary-containing tissue and the desired cells would be grown in culture in the presence of aFGF and any other supplemental components that might be required such as heparin and/or serum. After growth of  
10 adequate numbers of endothelial cells in culture to cover the synthetic polymeric blood vessel the cells would be plated on the inside surface of the vessel. Prior coating of the artificial vessel either covalently or noncovalently, with either heparin or  
15 proteins such as fibrin, collagen, fibronectin or laminin would be performed to enhance attachment of the cells to the artificial vascular surface. The cell-lined artificial vessel would then be surgically implanted into the patient and, being lined with the  
20 patients own cells, would be immunologically compatible. The non-thrombogenic endothelial cell lining should decrease the incidence of clot formation on the surface of the artificial vessel and thereby decrease the tendency of vessel blockage or  
25 embolism elsewhere.

The novel method is also useful for the production of artificial vessels. Vascular endothelial cells and smooth muscle cells from the patient would be obtained and grown separately in  
30 culture. The endothelial cells would be grown in the presence of the aFGF as outlined above. The smooth muscle cells would be grown in culture by standard techniques. A tubular mesh matrix of a biocompatible



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polymer (either a synthetic polymer, with or without a coating of either heparin or specific attachment proteins, or a non-immunogenic biopolymeric material such as surgical suture thread) would be used to

5 support the culture growth of the smooth muscle cells on the exterior side and vascular endothelial cells on the interior surface. Once the endothelial cells form a confluent monolayer on the inside surface and multiple layers of smooth muscle cells cover the

10 outside, the vessel is implanted into the patient.

The novel method can also be used for the induction of vascular growth. The pure growth factor or the equivalent human protein would be used to induce and promote the growth of blood vessels in the

15 patient. The mitogen would be administered along with any necessary stabilizers and enhancers of activity including heparin, at the site of desired vascular growth. For applications involving neovascularization of surface wounds, such as

20 abrasions or burns, the formulation would be applied directly in a slow release polymer at a rate of about 1-100 ng/day/cm<sup>2</sup> of injured surface. For internal vascular growth, the formulation would be released directly into the region to be neovascularized either

25 from implanted slow release polymeric material or from slow release pumps. The release rate in either case is preferably about 100 ng - 10 ug/day/cm<sup>3</sup> of injured tissue.

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EXAMPLE 1MITOGENIC RESPONSE OF FETAL BOVINE THORACIC  
AORTIC ENDOTHELIAL CELLS TO aFGF

5 Fetal bovine thoracic aortic endothelial  
cells (AG4762, N.I.A. Aging Cell Repository,  
Institute for Medical Research, Camden, New Jersey)  
were assayed after 38 cumulative population doublings  
10 in vitro. The cells were plated in 6-well Costar  
plates at  $2 \times 10^3$  cells/cm<sup>2</sup> in 20% heat  
inactivated fetal calf serum in Dulbecco's modified  
Eagle's medium (DMEM, Gibco) and changed to 1% serum  
18 hours later. All media were supplemented with  
glutamine and penicillin-streptomycin as previously  
15 described. Either pure aFGF diluted in 100 ul of 1  
mg bovine serum albumin (Sigma) per ml of DMEM or  
serum samples were added to each well along with 1.6  
uCi of <sup>3</sup>H-thymidine (New England Nuclear) and 45 ug  
of unlabeled thymidine in 40 ul of DMEM. After a 48  
20 hour incorporation period, the cells were washed,  
lysed and 75% of the trichloroacetic acid  
(TCA)-insoluble DNA from pure growth factor (-●-) or  
serum (-■-)-stimulated cells was counted.

The increase in endothelial cell population  
25 at various concentrations of aFGF was measured by  
measuring the uptake of tritiated thymidine. The  
results are shown in Figure 1, below.

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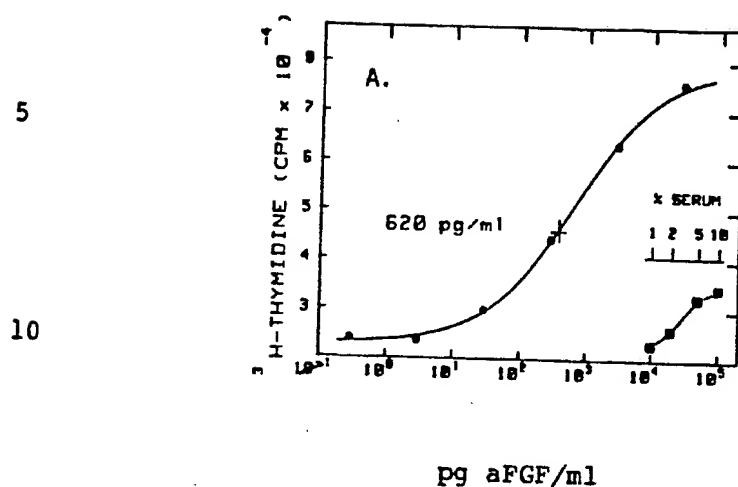


Figure 1

EXAMPLE 2MITOGENIC RESPONSE OF MOUSE LUNG CAPILLARY  
ENDOTHELIAL CELLS TO aFGF

20 Mouse lung capillary endothelial cells were  
 plated at  $2.6 \times 10^4$  cells/cm<sup>2</sup> in 0.5 ml/well in  
 24-well Costar dishes and grown to confluence in 10%  
 charcoal-treated calf serum (HyClone Laboratories,  
 Logan, Utah) in DMEM, lowered to 0.5% serum after 72  
 25 hours and allowed to become quiescent over 48 hours.  
 Either serum or the pure aFGF were added in 50 ul as  
 described above followed 18 hours later by a 4 hour  
 pulse of <sup>3</sup>H-thymidine (20 ul of 100 uCi/ml  
<sup>3</sup>H-thymidine in Gibco phosphate buffered saline).  
 30 The cells were processed and radioactivity counted as  
 described in Example 1, and the results were as shown  
 in Figure 2.

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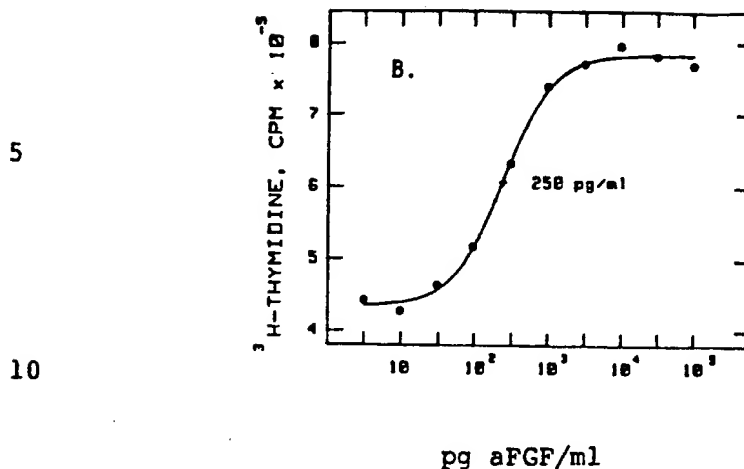


Figure 2

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EXAMPLE 3

Angiogenic Activity of aFGF

Chicken Egg Angiogenesis Bioassay

During the sustained vascular growth,

20 endothelial cells are observed to actively proliferate. Therefore, we tested the ability of the purified mitogen to induce blood vessel growth in the chicken egg chorioallantoic membrane angiogenesis assay. Based on previous reports that crude tumor

25 angiogenesis factor was significantly more active with coadministered heparin, we tested the vascularization response of heparin alone and heparin plus pure aFGF.

30 Three-day old chicken embryos were removed from their shells and grown in Handiwrap pouches suspended inside paper cups. The tops of the cups were covered with Handiwrap, and the eggs were incubated at 37°C in a tissue culture incubator for

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5-6 days. Either 1  $\mu$ g of pure aFGF in about 30  $\mu$ l of the HPLC elution solvent (7 mM trifluoroacetic acid/33% acetonitrile) or an identical HPLC solvent control solution were mixed with an equal volume of 2% low-gelling temperature agarose (Miles) dissolved in lactated Ringer's solution (Abbott) containing 10  $\mu$ g of heparin (from porcine intestinal mucosa; Sigma grade 1). Droplets (60  $\mu$ l) were allowed to gel on the center of sterile plastic 1.3-cm diameter Thermanox tissue culture coverslips (Miles), and at least part of the volatile acetonitrile evaporated by aeration for 15-30 minutes under a plenum of sterile air in a tissue culture hood. The coverslips were positioned, pellet down, over the chorioallantoic membrane of the eggs and incubated for 3 days. Eggs containing large white focal regions under the coverslips at the end of the assay, presumably formed by inflammatory cells, were discarded. The chorioallantoic membranes were examined microscopically and scored for the proliferation of fine capillaries under the center of cover-slips by observers who did not know the contents of the agarose pellets.

A 10 $\mu$ g dose of heparin per egg was inactive but the same amount of heparin plus 1  $\mu$ g of aFGF per egg appeared to enhance the growth of small capillaries at the site of application with no sign of inflammation (Table 1). The assay is reproducible, the results being a composite of three separate assays with different samples of aFGF. Control and positive angiogenic responses show the extent of capillary proliferation induced by aFGF. The mitogen is, therefore, a potent angiogenic protein in the presence of heparin.

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TABLE 1  
Angiogenic Activity of aFGF

	<u>Sample contents</u>	<u>Angiogenic response</u>	
		<u>Negative</u>	<u>Positive</u>
5	Control	15	0
	aFGF	2	10

These data are a composite of three separate experiments. Using t-distribution statistics, the group of mitogen-stimulated eggs ~~was~~ calculated to be different from the control population with a confidence level of 99.9%.

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WHAT IS CLAIMED IS:

1. A novel method for the stimulation of growth of vascular endothelial cells which comprises  
5 treating a sample of the desired endothelial cells in a nutrient medium with aFGF at a concentration of about 1-10 ng/ml.
2. A method for the stimulation of the  
10 growth of vascular endothelial cells in vitro which comprises treating a sample of vascular endothelial cells in a nutrient medium, comprising 2% or less of bovine serum, glutamine and a broad spectrum  
15 antibiotic with aFGF at a concentration of about 1-10 ng/ml.
3. The method of Claim 2 wherein cells from a vascular explant are grown on the surface of a synthetic polymeric vessel for implanting in the host.  
20
4. The method of Claim 2 wherein  
endothelial cells from a vascular explant are grown on the interior surface of a biocompatible tubular mesh support and smooth muscle cells are grown on the  
25 external surface of the tubular mesh support for implanting in the host.

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